

REMARKS

I. PRELIMINARY REMARKS AND CLAIM AMENDMENTS

With this response, claims 1 and 43 have been amended to eliminate the requirement that the hydroxycitric acid be bound to calcium and potassium as the present invention is not so limited. Dependent claims 8 and 50 reciting those preferred compositions have been reintroduced and have been amended to reflect that the preferred composition comprises a dual salt of HCA with calcium and potassium. In addition, the dependencies of claims 32, 42, 74 and 84 have been modified and claims 33, 34, 58, 59, 72, 73, 75 and 76 have been canceled to simplify the issues before the Examiner.

II. THE OUTSTANDING REJECTIONS

Claims 1, 9-11, 18, 25, 32-34, 36-43, 51-53, 58-60, 66,67, 72-76, 78-85 and 93-104 are pending in the application. The rejections to the claims are set out below.

Claims 1, 9-11, 18, 25, 32-34, 36-43, 51-53, 58-60, 66,67, 72-76, 78-85 and 93-104 stand rejected under 35 USC 103(a) as being unpatentable over Raju, WO99/03464 in view of one or more of Policapellio, US 5,612,039; Allen, US 5,480,657; Alvia et al., US 6,413,545 and Briggs et al., US 2004/0204472.

Claims 1, 9-11, 18, 24 (sic 25?), 43, 51-53, 58-60, 66, 67, 72, 73, 85 and 93-97 stand rejected under 35 U.S.C. §102(e) as being anticipated by Bhaskaran et al., US 2003/0207942.

Claims 85-104 stand provisionally rejected for obviousness-type double patenting over InterHealth's USSN 09/463,024.

III. PATENTABILITY ARGUMENTS

A. The Rejection under 35 U.S.C. §112(second paragraph) Should be Withdrawn.

The rejection for indefiniteness under 35 U.S.C. §112 (second paragraph) should be withdrawn in light of the amendment of claims 8 and 50 to recite that the preferred composition comprises a dual salt of HCA with calcium and potassium.

B. The Rejection under 35 U.S.C. §103(a) Should be Withdrawn because there is no teaching in the art that administration of HCA would reduce ghrelin levels.

The rejection over Raju, WO99/03464 in view of one or more of Policapellio, US 5,612,039; Allen, US 5,480,657; Alvia et al., US 6,413,545 and Briggs et al., US 2004/0204472 should be withdrawn because the claims are directed to methods for decreasing ghrelin levels in subjects in need thereof by administering sufficient amounts of hydroxycitric acid (HCA) and there is no teaching in the art that administration of HCA would reduce ghrelin levels!

Each of Raju, Policapellio and Alviar teach that HCA promotes weight loss and Allen teaches that niacin-bound chromium promotes weight loss but none of these references teach that HCA (or chromium for that matter) decrease ghrelin levels.

Further, Briggs does not mention HCA much less teach that HCA decreases ghrelin levels. Instead, Briggs is cited as teaching that “antagonists of ghrelin receptors [are] effective as weight loss agents.”

1. Briggs Teaches that Weight Loss Agents Can Have a Variety of Different Biological Activities Other than Ghrelin Receptor Antagonist Activity.

Briggs primarily teaches that COX-2 inhibitors are weight loss agents but does list ghrelin receptor antagonists among some 131 other weight loss agents for use in combination with COX-2 inhibitors.

Such weight loss agents include those with a wide variety of biological activities including (1) catecholamine modulators, (2) norepinephrine, dopamine and serotonin reuptake inhibitors, (3) lipase inhibitors, (4) dual serotonin reuptake inhibitors and serotonin releasing agents, (5) aminoketone class antidepressants, (6) activators of ATP-dependent K⁺ channels, (7) anti-hyperglycemics, (8) GABA enhancer and sodium channel blockers, (9) serotonin and dopamine releasers, (10) histamine-3 antagonists, (11) cannabinoid (CB1) receptor antagonists, (12) alpha adrenergic receptor agonists, (13) melanocortin-4 receptor agonists, (14) neuropeptide Y antagonists, (14) beta(3)-adrenergic

agonists, (15) glucagon-like peptide-1 agonists, (16) PPAR-gamma antagonists and PPAR-gamma partial antagonists, (17) urocortin agonists, (18) CCK agonists, (19) UCP activating agents, (20) prolactin modulators, (21) growth-hormone secretagogues, (22) ciliary neurotropic factors, (23) antihistamines, (23) 5-HT_{2C} agonists, (24) 5-HT_{2A} agonists, (25) dopamine agonists, (26) adipocyte complement-related protein (Acrp30) modulators, (27) cannabinoid antagonists, (28) tyrosine phosphatase modulators, (29) 11beta hydroxysteroid dehydroxysteroid dehydrogenase type 1 modulators, (30) cyclic AMP response element-binding protein modulators, (31) diacylglycerol o-acyltransferase modulators, (32) fatty acid transport protein 4 modulators, (33) G protein beta-3 subunit 825T modulators, (34) high mobility group 1C modulators, (35) Kallikrein modulators, (36) melanin-concentrating hormone receptor modulators, (37) perilipin modulators, (38) Tub gene modulators, (39) anticonvulsants, (40) leptin receptor modulators, (41) metabolic accelerators, (42) adipogenesis modulating agents, (43) HK-a receptor antagonists, (44) PPAR-gamma antagonists, (45) PPAR-alpha agonists and (46) leptin agonists.

It is presumed that the Examiner is not arguing that all weight loss agents have all of the 46 activities listed above so that any given weight loss agent, such as HCA, would necessarily have ghrelin receptor antagonist activity. However, if that is not the Examiner's argument, then which activity (or activities) of the forty-six (46) listed would HCA be expected to have? Where is the teaching in the art that instructs one that HCA has ghrelin receptor antagonist activity instead of some other activity such as growth-hormone secretagogue activity or adipocyte complement-related protein (Acrp30) modulator activity or is an antihistamine or has any of a variety of other activities?

In the absence of some teaching (which has yet to be cited to) it becomes clear that the identification of ghrelin receptor antagonist activity is made only in hindsight based on Applicants' own disclosure. There is no evidence that a ghrelin receptor antagonist activity would have been predicted for HCA from an examination of the art prior to Applicants' invention. Accordingly, the rejection should be withdrawn.

2. Applicants Do Not Claim that HCA is a Ghrelin Receptor Antagonist!

What makes it particularly clear that hindsight is in play as the basis for the obviousness rejection is that Applicants' claims are not directed to "ghrelin receptor antagonist activity"! Instead, Applicants have demonstrated that administration of HCA reduces ghrelin levels! This is quite different from acting as a receptor antagonist. Indeed, one of ordinary skill in the art might just as readily predict that a presumed receptor antagonist would act to increase ghrelin levels (perhaps through a feedback effect) rather than decrease ghrelin levels. For this reason, the statement that "[d]ecreasing ghrelin levels is deemed to be an inherent property" is incorrect, not least of all because affecting a receptor (as a receptor antagonist) does not necessarily have any effect on the receptor's binding pair. Further, the citation to *Atlas Powder* is inapplicable because Applicants are not claiming a composition but rather a method.

For these reasons, the applied references would not teach one that administration of HCA would decrease ghrelin levels as recited and accordingly, the obviousness rejection based on the combination of Raju, Policapellio, Allen, Alvia and Briggs should be withdrawn.

C. The Rejection over Bhaskaran under 35 U.S.C. §102(e) Should be Withdrawn.

The anticipation rejection over Bhaskaran US 2003/0207942 should also be withdrawn because while Bhaskaran teaches administration of HCA to overweight subjects 1) most overweight subjects have low ghrelin levels (and would not appear to be in any need of reducing their ghrelin levels) and 2) many subjects with high ghrelin levels are not overweight.

1. Most (but not all) Overweight Subjects Have Low Ghrelin Levels and it is Unexpected that HCA Administration Would Lower Ghrelin Levels.

In general, overweight individuals are characterized by low ghrelin levels compared to normal weight individuals and underweight individuals, such as those suffering from anorexia nervosa, have high plasma ghrelin levels. Ghrelin, <http://arbl.cvmbs.colostate.edu/hbloods/pathphys/endocrin/gi/ghrelin.html> (Attached hereto as Exhibit A) Thus, to the extent that Bhaskaran teaches administration of HCA to overweight subjects it would not be expected that HCA would lower ghrelin levels.

Moreover, not all (or even most) overweight subjects have high ghrelin levels and it is not clear that all overweight subjects need their ghrelin levels reduced. While many overweight subjects have low ghrelin levels (e.g., subjects suffering from nonalcoholic fatty liver disease tend to be overweight but have low ghrelin levels. See Marchesini et al., J. Clinical Endocrinology and Metabolism, vol. 88, No. 12, 5674-5679 (2003) (Attached hereto as Exhibit B)) others such as those suffering from Prader-Willi syndrome have plasma ghrelin levels that are “exceptionally high in comparison to patients similarly obese.” (Exhibit A).

It is therefore unclear whether administration of HCA to the class of overweight subjects generally or overweight subjects suffering from nonalcoholic fatty liver disease will decrease ghrelin levels. In any event, there is no evidence that administration of HCA to overweight subjects in the prior art might not have invariably decreased ghrelin levels because the ghrelin levels might not be high and such invariability is necessary for inherent anticipation.¹ . Nevertheless, and in its absence, the rejection under 35 USC 102 must be withdrawn.

¹ Inherency may not be established by probabilities or possibilities. It is necessary for inherency that the missing subject matter “is necessarily present in the thing described in the reference ...it may not be established by probabilities or possibilities... The mere fact that a certain result may result from a given set of circumstances is not sufficient.” MPEP 2163.07(a) *In re Robertson*, 49 USPQ 2d 1949, 1950-51 (Fed. Cir. 1999)

2. Many Subjects with High Ghrelin Levels are Not Overweight.

Similarly, many if not all subjects with high ghrelin levels are not overweight. Bhaskaran claims methods of reducing body weight by administering its HCA (claim 24) but it is widely known that those suffering from anorexia nervosa have high plasma levels of ghrelin. Misra et al., Am. J. Physiol Endocrinol Metab (March 8, 2005) attached hereto as Exhibit C. Additionally, ghrelin levels also tend to be high in patients who have cancer-induced cachexia. Wolf, et al., Cancer 106(4):966-73 (Feb 15, 2006) Abstract attached as Exhibit D.

Thus, any presumption based on Bhaskaran that a reduction in ghrelin levels will lead to weight loss or that weight loss is associated with a reduction in ghrelin levels is unsupported and contrary to the teachings of the art. Moreover, the discovery that HCA decreases ghrelin levels is of therapeutic interest beyond being a possible mechanism for weight loss in overweight individuals and could be of use in the treatment of non-overweight individuals suffering from high ghrelin levels, including possibly patients with cancer-induced cachexia or individuals suffering from anorexia nervosa.

For these reasons, the ability of HCA to reduce ghrelin levels is neither obvious in light of the prior art nor is it inherently anticipated by the teachings of that art and the rejections under 35 U.S.C. §102 and 103 should be withdrawn.

D. The Rejection for Obviousness-Type Double Patenting Should be Held in Abeyance.

The provisional rejection of claims 85-104 for obviousness-type double patenting over copending US. Serial No. 09/463,024 should be held in abeyance pending the determination of patentability of one or the other application. Claims 1-2, 13, 15-16, 24, and 26-28 stand rejected under 35 U.S.C. §102(b) over the '692 patent.

IV. CONCLUSION

In view of the above amendment and remarks, applicants believe the pending application is in condition for allowance. Should the Examiner wish to discuss any issues of

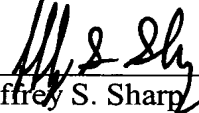
Application No. 10/805,129
Amendment dated September 4, 2007
After Final Office Action of March 8, 2007

Docket No.: 31174/30019

form or substance in order to expedite allowance of the pending application, she is invited to contact the undersigned at the number indicated below.

Dated: September 4, 2007

Respectfully submitted,



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Application No. 10/805,129
Amendment dated September 4, 2007
After Final Office Action of March 8, 2007

Docket No.: 31174/30019

EXHIBIT A



Ghrelin

EXHIBIT A

Structure of Ghrelin and Its Receptor

Ghrelin is synthesized as a preprohormone, then proteolytically processed to yield a 28-amino acid peptide. An interesting and unique modification is imposed on the hormone during synthesis in the form of an n-octanoic acid bound to one of its amino acids; this modification is necessary for biologic activity.

Synthesis of ghrelin occurs predominantly in epithelial cells lining the fundus of the stomach, with smaller amounts produced in the placenta, kidney, pituitary and hypothalamus.

The ghrelin receptor was known well before ghrelin was discovered. Cells within the anterior pituitary bear a receptor that, when activated, potentially stimulates secretion of growth hormone - that receptor was named the **growth hormone secretagogue receptor** (GHS-R). The natural ligand for the GHS-R was announced in 1999 as ghrelin, and ghrelin was named for its ability to provoke growth hormone secretion (the suffix ghre means "grow").

Ghrelin receptors are present on the cells in the pituitary that secrete growth hormone, and also have been identified in the hypothalamus, heart and adipose tissue.

Control and Physiologic Effects of Ghrelin

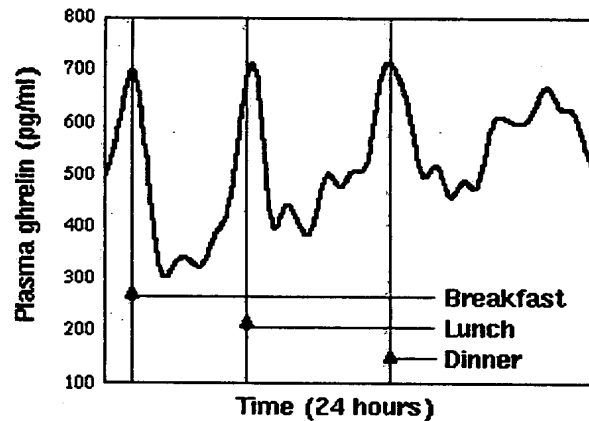
At least two major biologic activities have been ascribed to ghrelin:

- **Stimulation of growth hormone secretion:** Ghrelin, as the ligand for the growth hormone secretagogue receptor, potentially stimulates secretion of growth hormone. The ghrelin signal is integrated with that of growth hormone releasing hormone and somatostatin to control the timing and magnitude of growth hormone secretion.
- **Regulation of energy balance:** In both rodents and humans, **ghrelin functions to increase hunger** though its action on hypothalamic feeding centers. This makes sense relative to increasing plasma ghrelin concentrations observed during fasting (see below). Additionally, humans injected with ghrelin reported sensations of intense hunger. Ghrelin also appears to **suppress fat utilization in adipose tissue**, which is somewhat paradoxical considering that growth hormone has the opposite effect. Overall, ghrelin seems to be one of several hormonal signals that communicates the state of energy balance in the body to the brain.

Other effects of ghrelin include stimulating gastric emptying and having a variety of positive effects on cardiovascular function (e.g. increased cardiac output). It is not totally clear whether the cardiovascular effects are a direct effect of ghrelin or represent an indirect effect of ghrelin's ability to stimulate growth hormone secretion.

Blood concentrations of ghrelin are lowest shortly after consumption of a meal, then rise during the fast just prior to the next meal. The figure to

the right shows this pattern based on assays of plasma ghrelin in 10 humans during the course of a day.



Adapted from Cummings et al. Diabetes 50:1714, 2001.

Given the effects of ghrelin on energy metabolism and hunger, it is a prominent target for development of anti-obesity treatments. It has been reported that immunization of rats against ghrelin resulted in decreased weight gain and adiposity relative control rats, even though both groups consumed an equivalent amount of food. This intriguing experiment suggests the possibility of a vaccine against obesity.

Disease States

Ghrelin concentrations in blood are reduced in obese humans compared to lean control subjects, but whether this is cause or effect is not defined. Patients with anorexia nervosa have higher than normal plasma ghrelin levels, which decrease if weight gain occurs.

Prader-Willi syndrome is another disorder relevant to ghrelin science. Affected patients develop extreme obesity associated with uncontrollable and voracious appetite. The plasma ghrelin levels are exceptionally high in comparison to patients similarly obese due to other causes. Prader-Willi syndrome is clearly a complex disease with many defects; it may be that excessive ghrelin production contributes to the appetite and obesity components.

Index of: Gastrointestinal Hormones

◀ Secretin

Motilin ▶

Last updated on September 3, 2006

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EXHIBIT B

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Low Ghrelin Concentrations in Nonalcoholic Fatty Liver Disease Are Related to Insulin Resistance

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▶ Abstract

Several physiological and pathophysiological conditions, including changes in body fat, food intake, and insulin resistance, are known to be associated with variations in plasma ghrelin concentrations. We tested the hypothesis that insulin resistance exerts a primary role by measuring ghrelin in 86 patients with nonalcoholic fatty liver disease (NAFLD), a condition in which insulin resistance is relatively independent of obesity. Compared with 40 matched healthy subjects, patients with NAFLD had similar glucose levels and higher plasma insulin and insulin resistance [homeostasis model assessment (HOMA)-R index] by over 60%. Ghrelin was reduced (mean \pm SD, 226 ± 72 pmol/liter in NAFLD vs. 303 ± 123 in controls; $P < 0.0001$). In relation to quartiles of body mass index, ghrelin progressively decreased in

- ▲ [Top](#)
- [Abstract](#)
- ▼ [Introduction](#)
- ▼ [Patients and Methods](#)
- ▼ [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)

controls ($P = 0.003$), but not in patients ($P = 0.926$). In relation to quartiles of HOMA-R, ghrelin decreased in both groups, and significantly correlated with HOMA-R. After adjustment for age and sex, HOMA-R was the sole factor significantly associated with low ghrelin in the whole group (odds ratio, 5.79; 95% confidence interval, 2.62–12.81; $P < 0.0001$) and specifically in NAFLD (2.96; 1.12–7.79; $P = 0.028$). The study suggests that insulin resistance is a major factor controlling ghrelin levels in subjects with and without NAFLD.

► Introduction

GHRELIN IS A novel peptide involved in food intake and energy balance. Animal models have shown that ghrelin promotes energy sparing, finally leading to increased body weight. When injected in both humans and animals, ghrelin stimulates hunger (1), thus increasing food intake. In relation to these orexigenic properties, ghrelin has been extensively investigated as a possible cause of obesity. Surprisingly, the circulating levels of ghrelin are low in obese subjects when compared with normal controls (2), and increase sharply after weight loss (3). By contrast, ghrelin levels are elevated in constitutionally thin subjects with low body mass index (BMI) (4). The relative roles of either total body fat or associated metabolic conditions in regulating ghrelin concentrations have never been clarified. Increased body fat is generally associated with hyperinsulinemia and insulin resistance, and a correlation was reported between insulin levels or quantitative measures of insulin resistance and ghrelin concentrations in normal (5) and pathological conditions (6).

Nonalcoholic fatty liver disease (NAFLD) is a complex metabolic condition in which both lifestyle and genetic factors have a pathogenic role (7). It has been convincingly associated with the metabolic insulin resistance syndrome; most patients are overweight or frankly obese, with altered glucose regulation, dyslipidemia, and raised blood pressure, all contributing to the disorder (8). However, large studies have shown that approximately 10–20% of patients are lean and have normal glucose regulation, but are nonetheless insulin resistant when tested by the homeostasis model assessment (HOMA) method (9) or by the euglycemic clamp technique (10).

In the present study, we measured fasting ghrelin concentration in a large series of NAFLD patients with different phenotypes to test the relative importance of body fat, glucose regulation, hyperinsulinemia, and insulin resistance in ghrelin levels.

► Patients and Methods

Patients

Eighty-six NAFLD patients (79 males) (median age, 38 yr; range, 19–74 yr) and 40 control subjects (32 males) (median age, 43 yr; range, 28–77 yr) were included in the study. Their clinical and laboratory variables are presented in Tables 1 and 2. In NAFLD cases, the diagnosis was based on chronic hypertransaminasemia (alanine transaminases (ALT) of >1.5

- ▲ [Top](#)
- ▲ [Abstract](#)
- [Introduction](#)
- ▼ [Patients and Methods](#)
- ▼ [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)

- ▲ [Top](#)
- ▲ [Abstract](#)
- ▲ [Introduction](#)
- [Patients and Methods](#)
- ▼ [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)

times the upper normal values for 3 months or more), negative hepatitis B and C viral markers, absence of autoantibodies indicative of autoimmune hepatitis or celiac disease, negative or negligible alcohol consumption (<140 g/wk), and bright liver at ultrasound scanning. The diagnosis was confirmed by liver biopsy in 62 cases. According to the criteria proposed by Brunt *et al.* (11), 46 cases were classified as nonalcoholic steatohepatitis (NASH), and 16 were classified as pure fatty liver. Control subjects were free of hepatic and endocrine diseases. They were selected in a BMI range similar to that of NAFLD cases. Previously diagnosed diabetes mellitus [American Diabetes Association classification (12)] was an exclusion criterion for both NAFLD patients and control subjects. In 54 NAFLD patients and 28 controls, an oral glucose tolerance test (OGTT) was also performed for a complete evaluation of glucose tolerance.

View this table: TABLE 1. Phenotypic data of NAFLD patients and control subjects

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View this table: TABLE 2. Biochemical and clinical data of NAFLD and control

[\[in this window\]](#) subjects (mean \pm SD)

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All patients were regularly followed as outpatients and were on a controlled dietary regimen, comprising 25 kcal/kg body weight per day at the time of study. Blood samples for ghrelin concentrations were collected between 0800 and 0900 h, after an overnight fast. Plasma was immediately separated and stored at -80 C until analysis.

The purpose of the study was explained to all subjects, who gave their informed consent to blood sampling for ghrelin measurement. All other investigations were carried out during regular follow-up of NAFLD patients, according to specific protocols. The study was approved by the senior staff committees of the two university hospitals, institutional review boards regulating noninterventional studies.

Methods

Anthropometry. Body weight was measured in light clothing and without shoes to the nearest half-kilogram. Height was measured to the nearest half-centimeter. BMI was calculated as weight (kilograms) divided by height squared (square meters). Subjects with BMI between 25 and 30 kg/m² and greater than or equal to 30 kg/m² were considered overweight and obese, respectively. Waist circumference was measured at the nearest half-centimeter at the shortest point below the lower rib margin and the iliac crest, whereas hip circumference was similarly obtained at the widest point between hip and buttock. Body fat distribution was also evaluated by waist-to-hip ratio (WHR) according to World Health Organization (13).

Biochemical and hormonal measurements. Plasma immunoreactive ghrelin levels were measured in duplicate using a commercially available RIA (Phoenix Pharmaceuticals, Inc.,

Mountain View, CA) that uses ^{125}I -labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against the C-terminal part of human ghrelin (6). Intraassay and interassay coefficients of variation were less than 5.3 and 13.6%, respectively. This assay recognizes both acylated and deacylated ghrelin (2). The antiserum does not cross-react with any relevant peptide as previously shown (2, 14).

Plasma glucose was measured in duplicate with an automated analyzer. The coefficient of variation for any single determination was $\pm 1.5\%$. Insulin was measured by an immunoassay (AIA-PACK IRI, AIA-1200 system; Tosoh Co., Tokyo, Japan) with intraassay and interassay coefficients of variation for the quality control of less than 7%. Insulin resistance was calculated on the basis of fasting values of plasma glucose and insulin, according to the HOMA method (15) as follows: insulin resistance [HOMA-R (%)] = $\text{IRI}_0 \cdot \text{BG}_0 / 22.5$, where fasting insulin (IRI_0) is in microunits per milliliter, and glucose (BG_0) is in millimoles per liter.

In patients submitted to OGTT, two more indices of insulin sensitivity were derived from basal and postload glucose and insulin concentrations. The insulin sensitivity index (ISI) was calculated according to Matsuda and DeFronzo (16) as follows:

$$\text{ISI} = \frac{10,000}{\sqrt{(\text{BG}_0 \cdot \text{IRI}_0) \cdot (\text{average BG}_{0-120} \cdot \text{average IRI}_{0-120})}},$$

whereas the sensitivity index (SI) proposed by Cederholm and Wibell (17) was calculated as follows:

$$\text{SI} = \frac{75,000 + (\text{BG}_0 - \text{BG}_{120}) \cdot 0.19 \cdot \text{BW}}{120 \cdot \text{average BG}_{0-120} \cdot \log(\text{average IRI}_{0-120})},$$

where average BG_{0-120} and average IRI_{0-120} represent the mean of individual values measured after glucose load. Body weight (BW) also enters the SI equation, with a correction factor for glucose space. Additional correction factors are needed to transform metric units into SI units.

Fasting serum cholesterol, high-density lipoprotein (HDL) cholesterol, uric acid, and triglyceride levels were measured by routine laboratory techniques.

Statistical analysis

Data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Inc., Cary, NC.). Patients were grouped according to categorical variables (sex, class of BMI, presence/absence of impaired glucose regulation, and hypertension). Ghrelin concentrations were tested for significance using unpaired *t* test (two-tail) or nonparametric analysis (Mann-Whitney *U* test or Kruskal-Wallis test). Contingency test and Fisher's exact test were also used, whenever appropriate, to compare prevalence.

Logistic regression analysis was used to determine factors more closely associated with low ghrelin concentrations. For this purpose, a ghrelin concentration below the median of the whole

group (235 pmol/liter) was considered as dependent variable, and the clinical and laboratory values of Tables 1 and 2 were tested in univariate analysis. After this, variables significantly associated with low ghrelin were tested for independency in multivariate logistic regression analysis, after correction for age and sex. Two different models were built to further adjust data for BMI and waist circumference, separately.

All data in the text and in the tables are given as means \pm sd, when not otherwise indicated. Values of $P < 0.05$ were considered statistically significant.

► Results

Anthropometric and clinical data

Both controls and NAFLD patients were selected in a wide BMI range (controls, 19.2–35.2 kg/m²; NAFLD, 20.9–37.9 kg/m²), without differences in the distribution among BMI classes between groups. However, a larger waist circumference and a higher WHR, indicative of visceral adiposity, characterized NAFLD patients.

▲ Top
▲ Abstract
▲ Introduction
▲ Patients and Methods
• Results
▼ Discussion
▼ References

At history, diabetes, hypertension, and dyslipidemia were not more prevalent in NAFLD, but at the time of study, arterial pressure was higher in NAFLD, whereas HDL-cholesterol concentrations were lower. NAFLD patients were also characterized by a different response to OGTT. In particular, the prevalence of impaired glucose tolerance was remarkably higher in NAFLD (22.2 vs. 3.6% in controls; $P = 0.030$, Fisher's exact test) without differences in the prevalence of OGTT-detected diabetes (9.3% in NAFLD vs. 7.1% in controls; $P = 0.745$).

Fasting glucose was not different, but both insulin concentrations and HOMA-R were increased by over 60%. The 95% confidence interval of HOMA-R in normal-weight controls ranged up to 2.74%. This cutoff was used as the upper limit of normal insulin sensitivity. According to this criterion, 13 controls were considered to be insulin resistant (32%) vs. 57 of 86 NAFLD cases (66%; $P = 0.0005$, Fisher's exact test). A significant correlation was observed between BMI and HOMA-R in controls ($r = 0.334$; $P = 0.035$), but not in NAFLD patients ($r = 0.202$; $P = 0.062$).

Also, the two indices of insulin sensitivity derived by OGTT (ISI and SI) showed a significant resistance to insulin activity in the course of the glucose load in the NAFLD cohort when compared with control subjects.

Ghrelin levels

Ghrelin levels were reduced in NAFLD patients (226 ± 72 vs. 303 ± 123 pmol/liter in controls; $P < 0.0001$). Differences were maintained when levels were analyzed in relation to gender (males, NAFLD, 224 ± 74 pmol/liter, controls, 279 ± 100 pmol/liter, $P = 0.002$; females, NAFLD, 247 ± 35 pmol/liter, controls, 396 ± 164 pmol/liter, $P = 0.035$). Importantly, a gender effect was observed only in the control group (males vs. females, $P = 0.014$), whereas no statistically significant difference between males and females was observed in NAFLD.

When ghrelin levels were analyzed in relation to quartiles of BMI, in control subjects, ghrelin progressively decreased from 401 ± 130 (lower quartile; BMI range, 19.2–25.9 kg/m²) to 236 ± 75 (upper quartile; BMI range, 30.2–35.2 kg/m²; $P = 0.003$). In NAFLD patients, ghrelin levels were similar between subjects in the lower BMI quartile (242 ± 90 pmol/liter; BMI range, 20.9–24.6 kg/m²) compared with subjects in the upper quartile (244 ± 72 pmol/liter; $P = 0.926$; BMI range, 29.0–37.9 kg/m²) (Fig. 1[Ⓢ]). A significant correlation between BMI and ghrelin concentrations was present in controls ($r = -0.604$; $P < 0.0001$), but not in NAFLD ($r = -0.093$; $P = 0.397$) (Fig. 2[Ⓢ]).

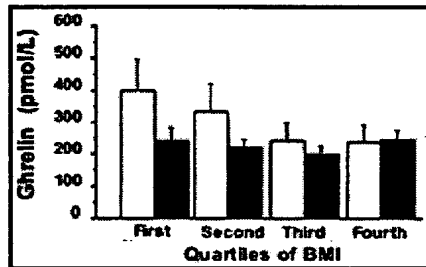


FIG. 1. Ghrelin concentrations (mean \pm 2 SE) in control subjects (\square) and in NAFLD patients (\blacksquare) in relation to quartiles of BMI. Significant differences are present in controls ($P = 0.0043$; Kruskal-Wallis test), but not in liver patients ($P = 0.083$).

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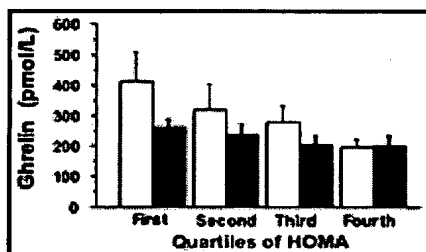
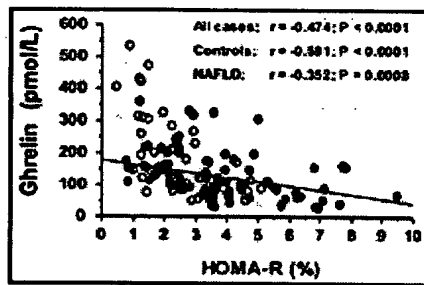


FIG. 2. Ghrelin concentrations (mean \pm 2 SE) in control subjects (\square) and in patients with NAFLD (\blacksquare) in relation to quartiles of the HOMA-R index. Significant differences are present both in controls ($P = 0.0009$; Kruskal-Wallis test) and in liver patients ($P = 0.0014$).

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In relation to quartiles of HOMA-R, a progressive decrease of average ghrelin concentrations was observed in both groups (Fig. 2[Ⓢ]), and the correlations between HOMA-R and ghrelin were both statistically significant (controls, $r = -0.591$, $P < 0.0001$; NAFLD, $r = -0.352$, $P = 0.0008$), although the two regression lines were considerably different, both in the intercept and in the slope (Fig. 3[Ⓢ]).

FIG. 3. Correlation between insulin resistance (HOMA-R) and ghrelin concentrations in control subjects (\circ , dotted line) and in patients with NAFLD (\bullet , continuous line). The r coefficients of correlation and P values are separately reported for the whole group ($n = 126$), for control subjects ($n = 40$), and for



fatty liver patients (n = 86).

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Less significant correlations were observed between ghrelin levels and other indices of visceral adiposity (waist circumference and WHR), as well as with indices of insulin sensitivity derived from OGTT (ISI and SI). In NAFLD patients, ghrelin failed to correlate with liver function tests (albumin and prothrombin time), as well as with liver cell necrosis (ALT, $r = 0.033$, $P = 0.766$).

Factors associated with ghrelin concentrations were further tested by logistic regression analysis (Table 3). Ghrelin concentrations below the median of 235 pmol/liter were associated with several laboratory and anthropometric indices at univariate analysis. The association of low ghrelin with both insulin and insulin resistance was maintained after adjustment for BMI, either in the whole population or in NAFLD patients (not reported in details).

View this table: **TABLE 3.** Factors associated with low ghrelin concentrations (<235 pmol/liter) at univariate and multivariate analysis

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After adjustment for age and sex, a HOMA-R indicative of insulin resistance ($\geq 2.74\%$) was the sole factor associated with low ghrelin concentrations at multivariate analysis in the whole population (Table 3), as well as separately in control subjects [odds ratio (OR), 18.90; 95% confidence interval, 3.52–101.48; $P = 0.0006$] and in NAFLD patients (OR, 2.96; 95% confidence interval, 1.12–7.79; $P = 0.028$). BMI did not enter the regression, and when data were adjusted for BMI, the effects of HOMA-R on ghrelin levels did not change (all cases, 5.58, 2.50–12.43, $P < 0.0001$; controls, 16.68, 2.80–99.28, $P = 0.002$; NAFLD, 2.83, 1.07–7.52, $P = 0.037$).

In the model in which data were further adjusted for waist circumference, in addition to age and gender, HOMA-R was similarly the sole factor significantly associated with low ghrelin, both in the whole population (OR, 6.00; 95% confidence interval, 2.42–12.96; $P < 0.0001$) and in NAFLD patients (2.92; 95% confidence interval, 1.08–7.85; $P = 0.034$).

NAFLD patients submitted to liver biopsy were not different from subjects who had not had a liver biopsy in the parameters presented in Table 2. In particular, BMI was $27.3 \pm 3.3 \text{ kg/m}^2$ in patients who had and $26.5 \pm 2.8 \text{ kg/m}^2$ in patients who had not had a liver biopsy ($P = 0.375$),

and HOMA-R was 3.89 ± 1.83 and $3.68 \pm 2.14\%$, respectively ($P = 0.673$). Also, ALT values were similar (biopsy positive, 84 ± 38 U/liter; biopsy negative, 73 ± 39 U/liter; $P = 0.294$). Ghrelin levels were also similar (234 ± 78 and 224 ± 71 pmol/liter; $P = 0.606$), and they did not differ in relation to the severity of fat deposition, fibrosis, and necroinflammatory activity. In particular, ghrelin was not different when patients were classified according to the presence/absence of NASH (pure fatty liver, 220 ± 88 pmol/liter; NASH, 226 ± 69 pmol/liter; $P = 0.781$).

► Discussion

Our data clearly show that ghrelin levels are reduced in NAFLD patients, after correction for sex, age, and BMI. Therefore, NAFLD may be included in the growing group of pathological conditions characterized by low ghrelin concentrations. The most likely reason for low fasting ghrelin is insulin resistance, which strictly correlates with ghrelin levels both when the two cohorts were examined together, and separately in NAFLD patients and in control subjects.

▲ Top
▲ Abstract
▲ Introduction
▲ Patients and Methods
▲ Results
• Discussion
▼ References

NAFLD is significantly associated with the metabolic syndrome (8). Although most patients are overweight or obese, 10–20% of cases have a BMI within normal limits. This condition represents a suitable model to study the relationship of insulin sensitivity and ghrelin, dissecting the putative effects of BMI on circulating ghrelin.

Our data are derived from a large cohort of NAFLD subjects, in whom insulin resistance, measured by the HOMA technique, was nearly the rule. The control group was carefully matched to account for potential confounders. Also, gender was considered, because a recent study demonstrated that ghrelin secretion is sexually dimorphic (18). Interestingly, our data showed that the gender effect is lost in NAFLD. Sex hormones may play an important role on ghrelin circulatory pattern (6, 19, 20), and estrogen therapy is a well-known cause of secondary NAFLD (21, 22). However, relative estrogen excess, as reported in advanced liver diseases, is unlikely in these subjects with normal liver function, and the result may simply stem from the low number of female patients causing type II statistical error.

To exclude differences related to BMI, our control population also included a few overweight or obese subjects with HOMA-R values in the range of insulin resistance as well. For this reason, ghrelin levels were analyzed in relation to quartiles of BMI and of insulin resistance, respectively. Similarly to data reported in other studies (23, 24), BMI played an important role on fasting ghrelin concentrations only in normal subjects.

By contrast, when data were analyzed in relation to quartiles of HOMA-R, the correlation between HOMA-R and ghrelin observed in controls was maintained in NAFLD, and HOMA-R was the sole factor predicting low ghrelin concentration in both groups at multivariate analysis.

The HOMA method for the measurement of insulin resistance has been extensively applied to epidemiological investigations. The figure of insulin resistance obtained with this method has a

relatively low reproducibility, which reflects day-to-day variability in fasting glucose and insulin, as well as analytical uncertainty. This is mainly the case for insulin levels, and a change of 1 $\mu\text{U/ml}$ insulin may determine a change of up to 20% HOMA-R. Despite this, the method proved to correlate closely with quantitative, functional tests such as the glucose clamp technique (25, 26). In nondiabetic subjects, insulin concentrations account for the larger part of HOMA-R. This explains why insulin *per se* was also closely associated with ghrelin. However, in logistic regression, HOMA-R was mathematically preferred to insulin, suggesting that insulin resistance rather than insulin concentrations may regulate ghrelin secretion.

Low ghrelin levels are observed in several pathological conditions characterized by insulin resistance, such as moderate to severe obesity (2), polycystic ovary syndrome (6), acromegaly (27), and primary or secondary hypogonadisms (20), but the reason(s) for such relations are unclear. Ghrelin stimulates food intake (28), and reduced levels might be teleologically aimed at preventing the increase in body mass. This conclusion holds in controls, but not in NAFLD patients, in whom ghrelin is relatively independent of BMI. There is no consensus as to the exact relationship between ghrelin and insulin. Ghrelin was reported to stimulate (29) as well as to inhibit insulin secretion (30); in turn, hyperinsulinemia, either induced by a single bolus or during a clamp, produces conflicting effects on ghrelin concentrations. *In vitro* animal studies (31) and human studies during insulin infusion (5, 32) support the role of insulin as a secretagogue of ghrelin, but this effect was not confirmed in similar experiments in humans (33, 34). In our study, ghrelin was inversely related to insulin, but HOMA-R values were the most significant predictor of low ghrelin concentrations. Accordingly, ghrelin secretion might be under the control of insulin or, more likely, under the control of insulin resistance, via undefined circulating factors.

Insulin resistance was also tested by means of OGTT-derived indices. Although an overall correlation was observed in controls, this relation was lost in NAFLD. The figure of insulin resistance derived from ISI and SI is largely dependent on the dynamic response of glucose and insulin to the glucose load, which is not necessarily related to fasting values. This is mainly the case of NAFLD patients, who had a larger prevalence of glucose intolerance. The assessment of ghrelin response to oral glucose in NAFLD would be needed to compare the dynamic responses of ghrelin and insulin to glucose ingestion, and to assess the role of postload insulin resistance.

In search of other potential factors responsible for low ghrelin levels, we correlated hormonal levels with liver function parameters, with negative results. Only one study has been published so far on ghrelin levels in patients with liver disease. Tacke *et al.* (24) reported normal ghrelin levels in noncirrhotic patients and slightly elevated concentrations in cirrhosis. In their series of patients with advanced disease evaluated for liver transplantation, ghrelin was only related to the clinical severity of disease. This correlation, however, might also be spurious, and generated by the anorexia and decreased food intake of advanced disease. In our NAFLD cases, liver function was normal, and all subjects were on a controlled dietary regimen. Their BMI was similar to controls, and also excessive food intake cannot account for hormonal changes.

In conclusion, the study of NAFLD patients, in whom insulin resistance is relatively independent of obesity, strongly supports a primary role of insulin resistance *per se* on fasting ghrelin

concentrations. This confirms the importance of decreased insulin sensitivity on the multiple metabolic abnormalities of patients with NAFLD.

► Footnotes

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Abbreviations: ALT, Alanine transaminase; BMI, body mass index; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; ISI, insulin sensitivity index; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OGTT, oral glucose tolerance test; OR, odds ratio; SI, sensitivity index; WHR, waist-to-hip ratio.

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▲ Top
▲ Abstract
▲ Introduction
▲ Patients and Methods
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• References

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EXHIBIT C

Secretary Dynamics of Ghrelin in Adolescent Girls with Anorexia Nervosa and Healthy Adolescents

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Ghrelin is an orexigenic peptide that is also a growth hormone (GH) secretagogue. Secretary dynamics of ghrelin have not been characterized in adolescent girls with anorexia nervosa (AN). We hypothesized that girls with AN would have increased ghrelin concentrations measured over a 12 hour period of nocturnal sampling compared with healthy adolescents as a consequence of increased basal and pulsatile ghrelin secretion, and that endogenous ghrelin secretory and concentration parameters would independently predict GH and cortisol. We performed overnight frequent sampling q 30 minutes over 12 hours in 22 girls with AN and 18 healthy adolescents 12-18 years old, and examined ghrelin concentration and secretory dynamics using Cluster and deconvolution analyses. Associations between ghrelin and GH, IGF-I, cortisol, leptin, total T3, luteinizing hormone (LH) and measures of insulin resistance were examined. Girls with AN were followed for one year and examined again at weight recovery (10% increase in BMI). On Cluster analysis, adolescent girls with AN had significantly higher ghrelin concentrations than healthy adolescents, including total area under the curve (AUC) for ghrelin ($p=0.002$), nadir ghrelin ($p=0.0006$) and valley ghrelin ($p=0.002$). On deconvolution analysis, secretory burst amplitude ($p=0.03$) and burst mass ($p=0.04$) were higher in girls with AN than in controls, resulting in higher pulsatile ($p=0.05$) and total ghrelin secretion ($p=0.03$) in AN. Basal ghrelin secretion and secretory burst frequency did not differ. Total ghrelin AUC predicted basal GH secretion ($r=0.49$, $p=0.002$), as did ghrelin burst mass, pulsatile and total ghrelin secretion. On multiple regression analysis, ghrelin concentration and secretion parameters were independent predictors of basal GH secretion.

Fasting ghrelin concentration was an independent predictor of GH burst frequency ($r=0.44$, $p=0.005$). The nutritional markers BMI and percent body fat predicted post glucose ghrelin levels ($r= -0.41$, $p=0.01$; and $r= -0.43$, $p=0.01$) and valley ghrelin ($r= -0.46$, $p=0.02$, $r= -0.43$, $p=0.03$) but not fasting ghrelin. Total ghrelin AUC was inversely associated with fasting insulin ($r= -0.50$, $p=0.002$) and HOMA-IR ($r= -0.54$, $p=0.0007$). Ghrelin parameters were also inversely associated with leptin and IGF-I. Insulin resistance was the most significant predictor of all ghrelin parameters. Ghrelin concentration was strongly and positively associated with cortisol concentration, and valley ghrelin was an independent predictor of cortisol burst frequency accounting for 52% of the variability. Ghrelin was also an independent predictor of total T3 and LH levels. We conclude that higher ghrelin concentrations in adolescent girls with AN are a consequence of increased secretory burst mass and amplitude resulting in increased pulsatile and total ghrelin secretion. The most important predictor of ghrelin concentration is the degree of insulin resistance, and ghrelin in turn predicts basal GH secretion, GH secretory burst frequency and cortisol burst frequency.

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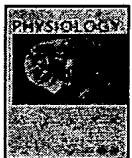
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R. Fernandez-Fernandez, M. Tena-Sempere, J. Roa, J. M. Castellano, V. M Navarro, E. Aguilar, and L. Pinilla

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Links

Adiponectin, ghrelin, and leptin in cancer cachexia in breast and colon cancer patients.**Wolf I, Sadetzki S, Kanety H, Kundel Y, Pariente C, Epstein N, Oberman B, Catane R, Kaufman B, Shimon I.**

The Institute of Oncology, Chaim Sheba Medical Center, Tel-Hashomer, Israel. wolf-i@inter.net.il

BACKGROUND: The hormone ghrelin and the adipocytokines leptin and adiponectin participate in body weight regulation. In response to weight loss, ghrelin and adiponectin levels increase and leptin decreases. Cancer cachexia is a complex metabolic state, characterized by loss of muscle mass and adipose tissue together with anorexia. The authors hypothesized that responses of these hormones may be attenuated in cancer cachexia. **METHODS:** Fasting plasma ghrelin, adiponectin, and leptin levels, as well as weight loss, were determined in 40 cancer patients: 18 of them suffered from cancer-induced cachexia, and 22 served as a comparison group. Hormone levels were measured before administration of cancer therapy. **RESULTS:** A similar distribution of age, gender, and diagnosis was observed in both study groups, but the cachectic patients had higher rates of metastatic disease and lower albumin levels. No significant correlation was observed between plasma adiponectin levels and weight loss. Mean plasma ghrelin levels were higher among cachectic compared with noncachectic patients. Notably, the association between ghrelin levels and weight loss was only modest, and in a third of the cachectic patients, ghrelin levels were equal to or lower than those in the noncachectic group. Plasma leptin levels showed gender-dependent associations, and significantly lower levels were found among cachectic women but not among cachectic men. **CONCLUSIONS:** Results suggested a gender-dependent attenuation of expected physiologic responses to weight loss among cancer cachexia patients. Thus, impaired response of adiponectin, ghrelin, and leptin may play a role in the pathogenesis of cancer cachexia syndrome. Copyright 2006 American Cancer Society.

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